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STRUCTURE of ANTIGENS

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PREFACE

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Chapter
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Molecular Dissection of Protein Antigens

Marc H. V. Van Regenmortel
Institut de Biologie Moléculaire et Cellulaire, CNRS
Strasbourg, France

ANTIGENIC DETERMINANTS OF PROTEINS

The antigenic specificity of a protein resides in restricted areas of the molecule, known as antigenic determinants or epitopes, which are recognized by the combining sites or paratopes of certain immunoglobulin molecules. The precision of steric and chemical fit between epitope and paratope necessary for achieving this type of immunological "recognition" is highly variable since different antibodies may have affinity constants in the range of 10^3 to 10^{11} L/mol.

The most common way of classifying epitopes consists in distinguishing continuous and discontinuous epitopes (Atassi and Smith, 1978). The label continuous epitope is given to any short linear peptide fragment of the antigen that is able to bind to antibodies raised against the intact protein. Usually these antibodies cross-react only weakly with the peptide and the continuous epitope identified in this manner is unlikely to mimic exactly the conformation and structure of the corresponding epitope in the intact protein. The peptide fragment probably does not retain the conformation present in the folded protein, and, furthermore, it is likely to represent only a portion of a more complex epitope.

The second type of epitope, known as discontinuous epitope, is believed to correspond to the vast majority of epitopes found in proteins. They are made up of residues that are n t continuous in sequence but are brought

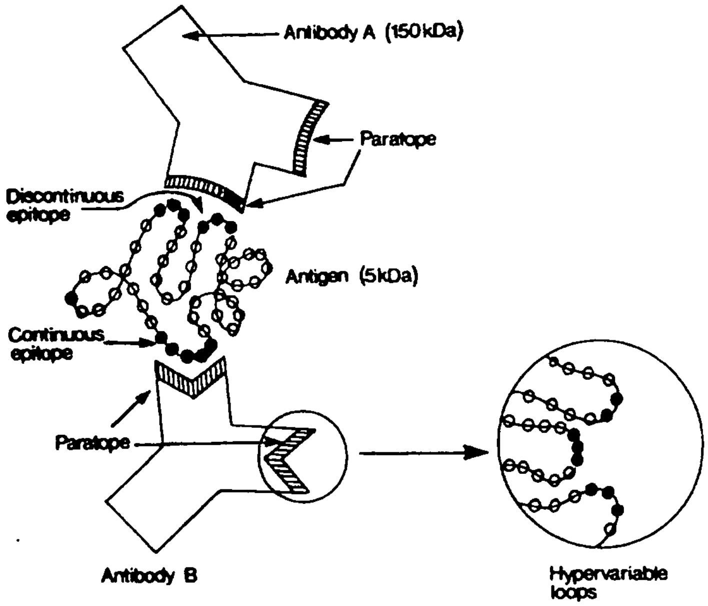

Figure 1. Schematic representation of two antibodies interacting with a continuous and a discontinuous epitope of a protein antigen. Contact residues are indicated in black. (From Van Regenmortel, 1986.)

together by the folding of the polypeptide chain (Fig. 1). Most antibodies to discontinuous epitopes will bind to the protein only if the molecule is intact and its conformation is preserved. When the protein is fragmented into a number of peptides, the various residues that made up the discontinuous epitope are scattered and each component is no longer individually recognized by the antibody. It is generally accepted today that the majority of monoclonal antibodies (MAbs) raised against intact proteins are specific for discontinuous epitopes, and it is believed that this explains why such MAbs usually do not react with any linear peptide fragment derived from the antigen. However, it is also true that a certain percentage of the MAbs raised against intact proteins, usually of the order of 10%, do react with linear peptide fragments of the protein. Since the range of specificities observed with a panel of MAbs is very similar to that found in a polyclonal antiserum to the same antigen (Quesniaux et al., 1990), it is reasonable to assume that about 10% of the antibodies present in an antiserum are also able to recognize so-called continuous epitopes of the protein antigen.

In recent years, some authors have challenged the view that *native* proteins possess continuous epitopes. Laver et al. (1990), for instance, suggested that all continuous epitopes represent "unfoldons," i.e., unfolded regions of the antigen that cross-react only with antibodies specific for the denatured protein. Such antibodies may be present in antisera raised against the protein because

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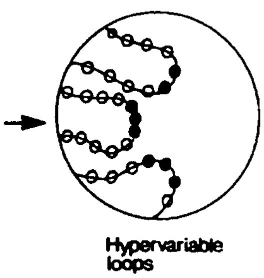

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some of the antigen molecules used for immunization were denatured (Scibienski, 1973; Lando and Reichlin, 1982; Jemmerson, 1987a). Similarly, antibodies obtained by immunization with linear peptides may also be specific for unfoldons and may recognize the parent protein only because of the presence of denatured protein molecules in the preparation used in the immunoassay. Although it is true that in many immunoassays in current use, the protein antigen is at least partly denatured, it seems nevertheless too extreme a view to maintain that all reported cases of cross-reactivity between proteins and peptides are due to antibodies specific only for the denatured form of the protein. The most compelling argument against this view lies in the ability of some antipeptide antibodies to neutralize certain biological activities associated with the native state of proteins. For instance, in the case of many different viruses, it has been firmly established that immunization with peptides can lead to the formation of antibodies that neutralize virus infectivity (Anderer and Schlumberger, 1965; Bittle et al., 1982; DiMarchi et al., 1986; Emini et al., 1985; McCray and Werner, 1987; Parry et al., 1988; Roehrig et al., 1989; Smyth et al., 1990). These findings imply that the antipeptide antibodies recognize the native state of the viral protein present in infectious particles. Such findings do not mean that the linear peptide reproduces exactly the epitope in the intact protein but only that it resembles the cognate structure in the parent protein sufficiently to allow antibody cross-reactivity.

Although only a small fraction of the total immune response against a protein leads to the formation of antibodies that cross-react with linear peptides, there is considerable interest in unravelling the structural basis of this cross-reactivity. The main impetus for these studies lies in the many practical applications that would arise if protein antigens could be replaced by synthetic peptides (Lerner, 1984; Walter, 1986).

ANTIGENIC CROSS-REACTIVITY

Antigenic cross-reactivity is a consequence of the fact that the relationship between an antigen and its antibody is never of an exclusive nature. Cross-reactivity may be observed because two multideterminant antigens share one or more identical epitopes recognized by distinct antibody subpopulations present in a polyclonal antiserum. This type of cross-reactivity, which may be described as *shared reactivity*, arises because a particular antibody recognizes the same epitope in two different proteins and is of little relevance in the present context (Berzofsky and Schechter, 1981). For instance, when a panel of MAbs raised against a multideterminant protein is tested against two antigenically related proteins, it is frequently observed that certain MAbs react in an identical fashion with the two antigens, obviously because they recognize the same epitope (Briand et al., 1982). A totally different type of cross-reactivity, which has been termed *true cross-reactivity* (Berzofsky and

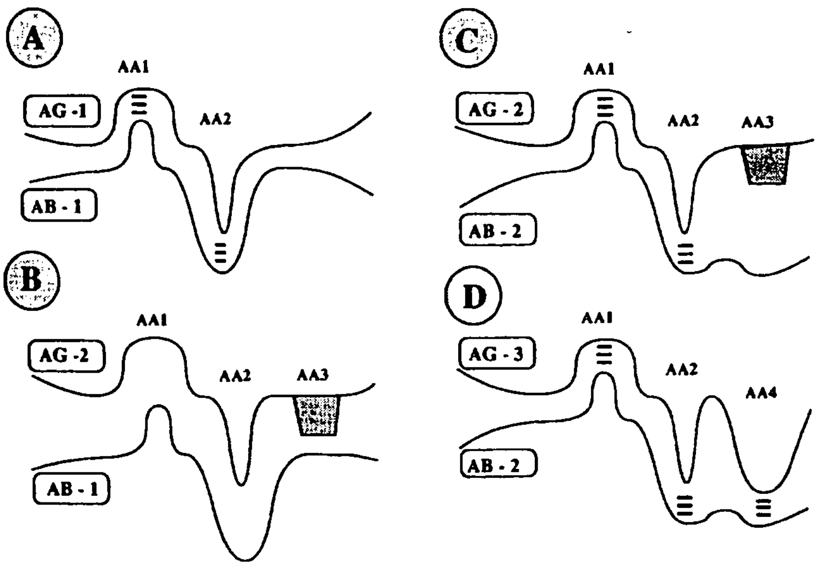

Figure 2. Schematic representation of epitope-paratope interfaces. In A, the energetic epitope of antigen 1 consists of two residues (AA1 and AA2) hydrogen-bonded to residues of the paratope of antibody 1. In B, the same energetic epitope of antigen 1 seems to be present, but a bulky substitution in a third residue (AA3) prevents H-bond formation with antibody 1. The influence of a substitution of AA3 might lead to the conclusion that this residue is implicated in the epitope of antigen 1, although its contribution is limited to the establishment of shape complementarity. In C, the energetic epitope in antigen 2 is again found to interact once the necessary complementarity in shape has been reestablished with antibody 2. In D, antibody 2 is able to show increased (i.e., heterospecific) binding with the modified epitope in antigen 3.

Schechter, 1981), arises when a particular antibody recognizes an epitope that is different but structurally related to the epitope used to raise the antibody. In most cases, the paratope will react with higher affinity with the homologous epitope used for immunization, although it is not uncommon for a paratope to bind more strongly to certain other epitopes than to the one against which it was raised (see Fig. 2). This phenomenon, known as heterospecificity (or heteroclitic binding), is commonly found whenever it is looked for, i.e., when the antibody is tested against a range of antigens closely related structurally to the immunogen (Al Moudallal et al., 1982; Harper et al., 1987; Mäkelä, 1965, Underwood, 1985).

True cross-reactivity occurs, for instance, when a peptide fragment of a protein cross-reacts with an epitope of the intact protein. Since the initial protein conformation present before fragmentation is unlikely to be retained in a peptide fragment, an antibody raised against the protein reacts with the peptide with a lower affinity. In addition, the linear peptide fragment usually represents only part of a more complex discontinuous epitope, which means that there will be fewer contact points between the paratope and the cross-

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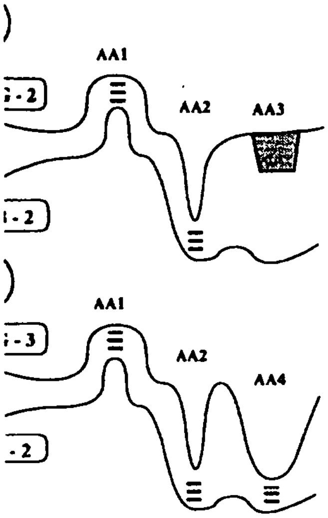

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reacting peptide than with the homologous protein. Whereas all six complementarity determining regions (CDR) of the antibody may have contributed some contact residues to the paratope that recognized the intact protein, it is possible that a smaller number of CDRs are involved in the binding observed with the cross-reactive peptide.

When there are only few contact points between paratope and epitope, the possibility arises that the CDRs that are not involved in the binding with a particular epitope will be able to bind to a totally dissimilar epitope. In this case the antibody, by the involvement of different subregions of the paratope, will be able to cross-react with two epitopes that have no structural similarity whatsoever. Such cross-reactions are probably responsible for the finding that MAbs sometimes react in a nonspecific manner with certain antigens. This reactivity is enhanced when the antigen presents a high local concentration of repeated epitopes or when it is used at a high density in solid phase or immunoblotting assays (Ghosh and Campbell, 1986). In both cases, the high local density of epitopes favors bivalent binding with IgG-type antibodies which facilitates the detection of weak cross-reactions shown by antibodies of low affinity. When IgM antibodies are used, the multivalence effect is further amplified (Hirayama et al., 1985), which makes irrelevant cross-reactions that are not based on a structural similarity in the epitope even more common (Ghosh and Campbell, 1986).

The study of cross-reactions between intact proteins and linear peptide fragments has led to the identification of large numbers of so-called continuous epitopes of proteins. Not every residue in the linear stretch of 5 to 8 residues that is believed to constitute a continuous epitope is, in fact, a contact residue interacting with the paratope. Usually some residues in the continuous epitope can be replaced by any of the other 19 amino acids without impairing the antigenic reactivity of the peptide (Geysen, 1985). This means that the linear peptide is, in fact, antigenically discontinuous and that the distinction between continuous and discontinuous epitopes is somewhat artificial (Van Regenmortel, 1987). There is no consensus at present about the minimum number of residues necessary for giving rise to a process that could properly be described as immunological recognition as opposed to chemical recognition. Obviously, the minimum number is one residue and there is, in fact, published evidence that a highly accessible, single C-terminal residue can be recognized with a certain degree of specificity by antibodies (Anderer and Schlumberger, 1966). This should come as no surprise in view of the existence of antibodies capable of recognizing as small a structure as dinitrophenol. There are 400 possible adjacent pairings of the 20 common amino acids, and studies by Geysen et al. (1986) have shown that certain MAbs are able to preferentially recognize some of these amino acid pairs. Since the immunoassay used to detect such antibody reactivity employs a high density of rod-coupled peptides, bivalent antibody binding is favored. This allows antibodies that have only a low intrinsic affinity for the dipeptide to be detected. When the dipeptide is extended at both ends by additi nal residues, some of the tetra- and hex-

apeptides that are obtained will be recognized by the MAb even better. When a particular residue is added to the dipeptide, it is sometimes found that it can be placed at more than one position and still increase the binding reaction. For instance, Geysen et al. (1986) reported that a MAb, which reacted weakly with the dipeptide Met-Lys, showed increasingly better reactivity with Try-Met-Lys, with Try-Met-Lys-His, and even better with Try-Gln-Met-Arg-His-Ser. This type of result implies that a variety of contacts can be established between epitope and paratope residues and that peptides of increasing size are able to readjust themselves to different subregions of the paratope (Edmundson et al., 1987).

In view of the multispecificity of antibodies, there is no guarantee that building up an epitope in this way (a "mimotope" according to Geysen's terminology) will produce a structure that is antigenically equivalent to the immunogen used to raise the test antibody. Until now there is no good evidence that immunization with such mimotopes produces antibodies able to recognize the structure being mimicked.

There is evidence that sequences of three residues can be recognized by certain antibodies of low affinity (Geysen et al., 1986; Trifilieff et al., 1991). It is also possible that longer peptides of 4 to 8 residues bind to an antibody because of the presence in the peptide of a few contact residues interspersed with noninteracting residues playing the role of scaffold. In such a case the systematic replacement of each residue of the peptide by other amino acids would show that the three contact residues are essential for binding while the other residues may be replaceable by virtually any amino acid (Schoofs et al., 1988). The minimum number of contacts or chemical bonds necessary for specific antibody recognition cannot be defined in an a priori manner. Furthermore, the minimum level of affinity required for calling the binding of an immunoglobulin "specific" and for considering that such an immunoglobulin is an "antibody" for the ligand depends on the context and on the type of assay used (Van Regenmortel, 1989a).

At the other end of the scale, it is equally meaningless to speak of perfect fit between epitope and paratope. It is clearly impossible to rule out that an antibody may show improved heterospecific binding with a structural relative of the epitope. In practice, it seems that *discrimination potential* is a more useful concept than *specificity* for describing the binding pattern of an antibody. It is the particular need of the investigator to differentiate between two entities that provides the necessary criterion for deciding whether an antibody is specific or not. Specificity is only meaningful with respect to a desired level of discrimination and this depends on the particular task at hand. The same antibody may thus be called specific or nonspecific, depending on the context.

Methods Used

Method	Ty Re
X-ray crystallography of antigen-Fab complexes	Dis re ge
Use of peptide fragments as cross-reactive antigenic probes 1. Free peptides 2. Peptides adsorbed to solid-phase 3. Peptides conjugated to carrier 4. Peptides attached to support used for synthesis	Co cr he
Identification of critical residues in peptide fragment by systematic replacement studies	Co cc re w di
Use of fusion proteins and peptides 1. Prokaryotic expression vectors 2. Chimeras	Co
Use of antipeptide antibodies	Co cı h
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TABLE 1.
Methods Used for Localizing Protein Epitopes

Method	Type of Epitope Recognized	Criterion for Residue Allocation
X-ray crystallography of antigen-Fab complexes	Discontinuous epitope reacting with homologous antibody	Contact with epitope-paratope interface
Use of peptide fragments as cross-reactive antigenic probes 1. Free peptides 2. Peptides adsorbed to solid-phase 3. Peptides conjugated to carrier 4. Peptides attached to support used for synthesis	Continuous epitope cross-reacting with heterologous antibody	Residual binding of linear fragment above threshold of assay
Identification of critical residues in peptide fragment by systematic replacement studies	Continuous epitope containing essential residues interspersed with irrelevant residues	Abrogation or decrease of cross-reactivity by replacement of functionally essential residue
Use of fusion proteins and peptides 1. Prokaryotic expression vectors 2. Chimeras	Continuous epitope	Residual binding above threshold of assay
Use of antipeptide antibodies	Continuous epitope cross-reacting with heterologous antibody	Induction of cross-reactive antibodies
Study of mutants, analogs and homologous proteins	Discontinuous epitopes	Abrogation or decrease of cross-reactivity
Topographic mapping by competitive binding assay	Only relative position of epitopes is defined	

METHODS USED FOR LOCALIZING EPITOPES

Methods used for identifying epitopes in proteins have been described in several reviews (Atassi, 1984; Benjamin et al., 1984; Berzofsky, 1985; Jemmerson and Paterson, 1986; Van Regenmortel, 1984, 1989a). The different approaches that have been used are summarized in Table 1. As discussed elsewhere (Van Regenmortel, 1989b), the only method that is truly based on a structural analysis is X-ray crystallography of antigen-antibody complexes. This analysis describes the spatial relationships observed at the two interacting surfaces and tends to emphasize the static aspects of the contact points once a stable complex has been formed. It should be noted that the identification

of a contact residue contributing to the structure of an epitope is not at all straightforward since different definitions of what constitutes a contact have been given (Getzoff et al., 1988). For instance, contact residues can be defined as residues within Van der Waals contact, residues buried to a certain radius probe sphere, or residues with their side chains interacting directly. The five epitopes that have so far been analyzed by X-ray crystallography were found to comprise 15 to 22 contact residues, a size considerably larger than what has been traditionally regarded as being the size of an epitope, i.e., 5 to 6 residues.

All other methods listed in Table 1 are based on binding measurements and introduce the fourth dimension of time as a component of what is being observed. Binding assays take the form of activity measurements and correspond to a functional analysis of antigen-antibody interaction (Van Regenmortel, 1989b). Such analysis incorporates dynamic aspects that are not directly perceptible in a structural picture describing the bound state at equilibrium. Furthermore, most methods based on binding assays listed in Table 1 analyze the cross-reactive binding properties of antibodies, i.e., the ability of antibodies to cross-react with related structures that may differ considerably in conformation from the intact homologous antigen used to raise the antibody. Therefore, much of the information that is obtained in this way relates to rather incomplete and adulterated versions of the original epitopes existing in the native protein. These methods have led to the conclusion that only 3 to 8 residues of the antigen are critical to antibody binding. The epitope defined in a functional sense thus appears to involve fewer residues than the epitope defined in structural terms. Recently, an attempt has been made to compute how many of the residues allocated to various epitopes actually contribute to the binding energy of interaction (Novotny et al., 1989). By calculating the relative binding contributions of individual residues in the epitope defined in structural terms, it was found that most of the free energy contribution came from as few as 3 to 5 residues. This makes it possible to define a so-called "energetic epitope" in which the energetics of complex formation are emphasized and which has a size similar to that of epitopes defined by binding assays. The additional residues present in the structural epitope can then be viewed as playing a scaffolding role necessary to keep the critical, interacting residues in their proper position and orientation.

An important conclusion, therefore, is that different analytical approaches lead to different perceptions of what constitutes a protein epitope. As illustrated schematically in Fig. 2, an energetic epitope may, for instance, be viewed as comprising two residues (AA1 and AA2 in Fig. 2A) which are hydrogen-bonded to residues of the paratope. Although both residues are retained in antigen 2 (Fig. 2B), a bulky substitution at AA3 located outside the energetic epitope would eliminate the binding potential of the energetic epitope for antibody 1. If the epitope had been mapped in terms of which substitutions affect binding, residue AA3 would have been included in the epitope of antigen 1. On the other hand, when tested with respect to antibody

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2, the energetic epitope in antigen 2 is again able to bind (Fig. 2C). Furthermore, another substitution (AA4 in antigen 3, Fig. 2D) may give rise to heterospecific binding by antibody 2. Clearly, epitope-paratope pairs are relational partners that should be defined only in terms of each other and not in the absolute. Like all relational concepts (e.g., father, brother) an epitope exists only by virtue of its relationship with a complementary partner, i.e., a particular paratope.

Crystallographic Analysis of Protein-Antibody Complexes

During the last 5 years, our knowledge of the structural basis of antigen-antibody interaction has been considerably advanced by X-ray diffraction studies of complexes of monoclonal antibody Fab fragments with their protein antigens (Colman, 1988; Davies et al., 1988; Mariuzza et al., 1987). Five epitopes have been analyzed by this method, three of lysozyme (Amit et al., 1986; Padlan et al., 1989; Sheriff et al., 1987) and two of influenza neuraminidase (Colman et al., 1987; Tulip et al., 1989). In all five cases, a large area of the protein surface (700 to 800 Å^2, comprising between 15 to 22 amino acid residues was identified as being in contact with residues of the antibody combining site. In all cases there was so much complementarity in shape between the interacting surfaces of antigen and antibody that water was almost entirely excluded from the interface (see Chapter 2).

All epitopes identified by X-ray crystallography so far are clearly discontinuous. The epitopes recognized by the antilysozyme D1.3 and HyHEL-5 antibodies consist essentially of two stretches of the lysozyme polypeptide chain (residues 18 to 27 and 116 to 129 for D1.3, and 41 to 53 and 67 to 70 for HyHEL-5), whereas the lysozyme HyHEL-10 epitope consists of the exposed surface of a helix (residues 88 to 99) together with residues from several lysozyme segments (residues 15 to 16, 20 to 21, 63, and 74 to 75). The two neuraminidase epitopes are also discontinuous. The NC41 epitope consists of segments 368 to 370, 400 to 403, 430 to 434 and portions of 325 to 350 (Colman et al., 1987), whereas the NC10 epitope involves five segments of the polypeptide chain.

The three lysozyme paratopes are made up of residues from all six CDRs of the antibody, whereas the two neuraminidase paratopes utilize residues from only four CDRs (antibody NC10) or five CDRs (antibody NC41). In addition, some framework residues are also involved in the binding interaction.

In the lysozyme-antibody complexes, no large conformational changes were observed in the lysozyme as a result of the binding to antibody. However, movement of the backbone atoms of as much as 2 Å at the point of contact have been recorded (Davies et al., 1988). It is a matter of debate whether such small adjustments are in favor of the "induced fit" mechanism proposed for antigen-antibody interaction or whether they are compatible with the "lock-and-key" model (Mariuzza et al., 1987; Van Regenmortel, 1989a). It is clear

that readjustment of side chains does occur but movement of the backbone atoms is more difficult to establish unambiguously. The magnitude of the motions observed in the segmental mobility of the peptide chain is only of the order of 1 t 2 Å and the functional significance of such small movements is a matter of interpretation. Recently, a small rearrangement (0.5 to 0.7 Å) of the V_H and V_L domains of the paratope of antilysozyme antibody D1.3 was found to occur upon complex formation with the antigen and this was interpreted in terms of induced fit (Bhat et al., 1990).

The two explanatory models of antigen-antibody interaction, the lock-and-key and induced fit models, are sometimes presented as mutually exclusive, although it is clear that both models are useful to describe some aspects of epitope-paratope recognition. In a similar vein, the opposition between static and dynamic views of antigenicity seems somewhat artificial (Novotny et al., 1987a). The debate on whether the location of epitopes in proteins is better "explained" by the static surface accessibility of certain regions or by their segmental mobility (Novotny et al., 1986; Sasaki et al., 1988; Tainer et al., 1985; Westhof et al., 1984) is clouded by the fact that the accessibility and mobility of short segments of polypeptide chains are not independent variables but represent interconnected aspects of the folding pattern of globular proteins. Loops and turns, for instance, are mostly surface projections and also tend to possess higher than average mobility. Attempts to find the best correlation between these properties and the antigenicity of ill-defined "continuous" epitopes have given rise to much debate and contradictory claims (Geysen et al., 1987a; Hopp, 1986; Novotny et al., 1987b; Thornton et al., 1986). Unfortunately, the search for correlations has not yet produced an effective method for predicting the location of epitopes in proteins.

Preliminary X-ray data of crystal structures of complexes of peptides with antipeptide Fab have been published (Schulze-Gahmen et al., 1988; Stura et al., 1989). In one study, the detailed structure of a complex between peptide 67 to 89 of myohemerythrin and a Fab fragment obtained by immunization with the peptide was presented (Stanfield et al., 1990). Surprisingly, the N-terminal part of the peptide was found to adopt a β-turn conformation in the antibody-peptide complex, although the same region in native myohemerythrin was in a helical conformation. However, since the Mab used to prepare the Fab had been screened for reactivity with solid-phase plate-bound myohemerythrin (Fieser et al., 1987) it is possible that the helical conformation was absent in the plate-bound antigen. The authors reported that solution-phase myohemerythrin was able to compete with the solid-phase protein in ELISA and suggested that this unusual finding was brought about by a conformational change due to "unknown causes" (Stanfield et al., 1990). It is not clear to what extent induced fit phenomena may be responsible for these observations (Crumpton, 1986; Wilson et al., 1985).

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Studies with Peptides as Antigenic Probes

The most widely used method for localizing protein epitopes consists in identifying which natural or synthetic peptide fragments of the molecule are able to cross-react with antibodies raised against the intact protein. Any peptide that is able to bind to the protein antibodies is said to contain a continuous epitope. Peptides of decreasing size may be tested to determine which is the smallest peptide that retains a significant level of antigenic reactivity (Benjamini, 1977). The degree of antigenic cross-reactivity observed with small peptides is mostly very low with the exception of peptides corresponding to chain termini. In a majority of proteins, the terminal segments are surface-oriented (Thornton and Sibanda, 1983) and are also more mobile than internal sections of the polypeptide chain. Both features may explain why the cross-reactivity observed with short terminal peptides tends to be much higher than with internal peptides (Absolom and Van Regenmortel, 1977; Milton and Van Regenmortel, 1979; Tainer et al., 1985; Walter, 1986; Westhof et al., 1984).

Free Peptides and Peptide Conjugates

As a result of rapid developments in the technique of solid-phase peptide synthesis (Atherton and Sheppard, 1989; Houghten, 1985; Kent and Clark-Lewis, 1985; Plaué, 1990; Van Regenmortel et al., 1988), synthetic peptides have virtually replaced natural peptide fragments as antigenic probes. After synthesis, the peptides are cleaved from the resin and are tested either as free peptides in solution, as immobilized peptides adsorbed to a solid phase, or as peptide-carrier conjugates. The different immunoassay formats used to determine the antigenic reactivity of peptides have recently been reviewed (Van Regenmortel et al., 1988). It should be stressed that the antigenic reactivity of peptides is highly dependent on the assay format (Muller et al., 1986). Sometimes the free peptide in solution is most active (Altschuh and Van Regenmortel, 1982; Nestorowicz et al., 1985), but in other cases the activity is higher when the peptide is conjugated to a carrier (Al Moudallal et al., 1985). In such cases the microenvironment at the surface of the carrier protein probably induces the peptide to adopt a more suitable conformation for antibody recognition. It should also be recognized that peptides corresponding to inner sequences of a polypeptide chain possess ionizable groups at their termini which are absent in the protein because of the formation of peptide bonds. The presence of the additional charged groups could lower any potential antigenic cross-reactivity with the intact protein. On the other hand, when the free α-carboxyl group of the terminal COOH is replaced by an amide group, the antigenicity of the peptide may also be altered drastically (Gras-Masse et al., 1986; Hodges et al., 1988). It must be recognized, therefore, that the detection of cross-reactivity with the parent protein can be adversely affected both if the extraneous charges of the peptide are retained or if the charges are removed by amidation or acetylation.

The binding of a peptide to antiprotein antibodies may be facilitated by the induction of a native-like conformation in the peptide during formation of the peptide-antibody complex (Crumpton, 1986; Getzoff et al., 1987, 1988). The use of longer peptides does not necessarily lead to a higher level of cross-reactivity since longer peptides may adopt a conformation different from that present in the native protein (Jemmerson, 1987b; Wilson et al., 1984). Shorter peptides may also fold more easily into the proper orientation required for binding to the antibody (Hodges et al., 1988). A variety of approaches have been used to increase the level of conformational mimicry between peptide and intact protein (see Chapter 3). Cyclization of the peptide has frequently been used for this purpose (Arnon et al., 1971; Dorow et al., 1985; Dreesman et al., 1982; Fourquet et al., 1988; Jemmerson and Hutchinson, 1990; Schulze-Gahmen et al., 1986), although it appears that information on the three-dimensional structure of the epitope is required to achieve the best results (Muller et al., 1990; Plaué, 1990). Other strategies for stabilizing certain peptide backbone conformations have also been proposed (Gras-Masse et al., 1988; Mutter, 1988; Satterthwait et al., 1989).

The antigenic cross-reactivity of peptides may be tested by measuring their capacity to inhibit the reaction of the protein with its homologous antibodies, or simply by adsorbing the peptides to a solid phase and measuring their ability to be recognized by antiprotein antibodies. In the latter case, it may be necessary to try a variety of buffers (Geerligs et al., 1988) and to prevent the peptide solution from drying up during the test (Norrby et al., 1987). It has been suggested that in order to establish the specific nature of a cross-reaction, it is necessary to include controls in which the liquid-phase protein is allowed to compete with plate-bound antigen and to show that inhibition to a level approaching 100% occurs (Jemmerson, 1987a). However, it cannot be excluded that a genuinely cross-reacting antigen reacts with antibodies so weakly that a complete inhibition cannot be observed at experimentally achievable concentrations (Berzofsky and Schechter, 1981).

Peptides Attached to Support Used for Synthesis

Peptides can be tested for antigenic activity without prior cleavage from the support used during synthesis (Hurrell et al., 1977; Shi et al., 1984). The pepscan method developed by Geysen et al. (1984, 1987b) allows the concurrent synthesis of hundreds of peptides on polyethylene pins. The pins are assembled into a polyethylene holder with the format and spacing of a microtiter plate. This allows the peptides to be tested by an enzyme immunoassay while they remain attached to the pin. After each test the pins can be freed of bound antibody by sonication and retested with different antibody preparations as many as 30 times. The pepscan method is ideally suited for the systematic testing of all possible overlapping peptides of a protein, starting from the N-terminal residue down to the C-terminal one. Usually 6 to 10 residue-long peptides are analyzed in this fashion (Geysen et al., 1987b). Covalent attachment of the peptide to the solid support may, in some cases,

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impair its antigenic activity and it is thus possible to miss certain peptides that would be revealed as antigenically active in a different type of immunoassay. Another problem encountered with the pepscan technique is that some peptide sequences tend to give rise to nonspecific binding. The high concentration of peptide on the pins favors bivalent binding of antibody and facilitates the detection of very low levels of cross-reactivity. As a result, di- or tripeptide sequences may give rise to observable cross-reactions in this technique (Geysen et al., 1986; Trifilieff et al., 1991).

Identification of Critical Residues by Replacement Studies

The pepscan technique is also frequently used to determine the contribution of individual amino acids to the binding interaction between peptide and antibody (Getzoff et al., 1988). This is achieved by analyzing peptide replacement sets in which each residue of the peptide is, in turn, replaced by the other 19 possible amino acids. In this way some residues are found to be essential for binding since they cannot be replaced by any residue without impairing the antigenic reactivity. Presumably they correspond to critical residues that contribute to the energy of interaction. Other residues can be replaced by all common amino acids without affecting binding and their role may be limited to that of a scaffold. By analyzing the pattern of replaceability linked to retention of antibody binding in 103 continuous epitopes of various proteins, Geysen et al. (1988) found that, on average, only five out of six residues in hexapeptides were essential for activity.

Studies with Fusion Proteins and Peptides

Epitopes can be identified by expressing parts of the protein in a prokaryotic expression system and measuring the antigenic activity of the expression product (Lenstra et al., 1990). For instance, in the pEX expression system, the product of an inserted DNA fragment becomes the C-terminal portion of a hybrid galactosidase protein which precipitates in the cell. The hybrid protein is solubilized in sodium dodecyl sulfate (SDS) and transferred to a nitrocellulose filter for testing its antigenicity (Stanley, 1983). In recent years a number of expression vector systems have been developed (Charbit, 1991; Mehra et al., 1986; Young and Davis, 1983). Viral chimeras, in which the epitopes of one virus are inserted by recombinant DNA techniques into the particles of another virus, are also increasingly used (Clarke et al., 1987; Delpeyroux et al., 1986; Michel et al., 1988). The recombinant approach to epitope analysis has been reviewed by Hofnung and Charbit (1992).

Studies with Antipeptide Antibodies

In these studies, synthetic peptides are used for immunization and the resulting antipeptide antibodies are tested for their ability to cross-react with the intact protein. A positive cross-reaction is taken as an indication that the

peptide approximates to an epitope of the protein. Alth ugh it has repeatedly been claimed (Green et al., 1982; Lern r, 1984; Niman et al., 1983) that immunization with peptides leads to a very high frequency of induction of antibodies able to recognize the *native* protein, it is now generally accepted that these claims arose because the antipeptide antibodies reacted with denatured protein molecules present in solid-phase immunoassay (Jemmerson, 1987a; Jemmerson and Blankenfeld, 1989; Van Regenmortel, 1989a). It is now widely recognized that proteins become at least partly denatured when they are adsorbed to plastic during a solid-phase assay (Darst et al., 1988; Friguet et al., 1984; Soderquist et al., 1980) which explains why antipeptide antibodies frequently react quite well with plate-bound protein antigens (see Chapter 11).

The contention that antibodies against a highly disordered state (the peptide) are mostly able to recognize the highly ordered state (the folded protein in its native conformation), although the reverse is not necessarily the case, has been called the order-disorder paradox (Dyson et al., 1988). In an attempt to resolve this paradox, it was suggested that a preferred conformation of the peptide present in solution becomes stabilized at the surface of the carrier protein or when the peptide binds to the B-cell receptor during immune stimulation. However, if a process of induced fit is able to influence the conformation of the immunogenic form of the peptide, one would expect that a similar induction of conformation would occur when the peptide interacts with antiprotein antibodies. In other words, antibodies against the ordered state should also be able to recognize at high frequency the disordered peptide, which was in fact not observed (Green et al., 1982). The paradox simply vanishes, however, if it is accepted that the extent of cross-reactivity between peptides and native protein is always rather low, irrespective of whether antiprotein or antipeptide antibodies are tested. This low level of cross-reactivity is illustrated by the finding that intact cytochrome c was able to activate only a small fraction of peptide-primed B lymphocytes (Jemmerson and Blankenfeld, 1989).

In order to produce antipeptide antisera, it is customary to couple peptides of less than 10 to 15 residues to a carrier protein (Briand et al., 1985; Palfreyman et al., 1984; Van Regenmortel et al., 1988). Since the method of conjugation may strongly influence the type of antipeptide antibodies that are produced (Bahraoui et al., 1987; Dyrberg and Oldstone, 1986; Mariani et al., 1987; Schaaper et al., 1989), it is advisable to use more than one conjugation procedure and immunization schedule. An approach which avoids the formation of antibodies to a carrier protein is the multiple-antigen peptide (MAP) system introduced by Tam (1988). The MAP consists of a core matrix made up of three levels of lysine residues and eight amino terminals for anchoring peptide antigens. Although the MAP system enhances the immunogenicity of peptides and leads to high levels of peptide antibodies in immunized animals, the antibodies do not always cross-react strongly with the cognate protein.

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Studies with Mutants and Analogs

In this method the antigenic cross-reactivity between related proteins presenting known amino acid substitutions is studied by means of MAbs. If the substitution leads to a change in antibody binding, it is assumed that the mutated residue is involved in the structure of an epitope (Hornbeck and Wilson, 1984). In general, it seems that single substitutions at the surface of a protein only cause a local change with no long-range structural alterations (Benjamin et al., 1984). This is demonstrated by the fact that usually other MAbs directed against neighboring epitopes of the protein are unaffected by the one substitution. There are exceptions, however, and certain mutations may alter the antigenicity by an indirect distal conformational effect (Al Moudallal et al., 1982; Barnett et al., 1989; Blondel et al., 1986; Collawn et al., 1988; Hurrell et al., 1977).

Since the number of available substitutions in a series of homologous proteins is always limited, a more extensive analysis may require generating protein variants by site-directed chemical modification (Cooper et al., 1987; Oertle et al., 1989) or by site-directed mutagenesis (Smith et al., 1991). This latter approach has the added advantage that multiple substitutions can be examined for any given amino acid. In this case, a residue is defined as a contact residue if the substitution leading to decreased binding does not significantly increase the side-chain volume (Smith and Benjamin, 1991). Such a precondition is introduced because substitution of a small noncontact residue by a bulky side chain could alter binding by preventing antibody from establishing normal bond distances with other contact residues (Fig. 2C).

Instead of inferring the position of epitopes from the ability of MAbs to distinguish between protein variants, it is possible in the case of infectious agents to select nonneutralizable mutants by the immunological selection pressure of neutralizing MAbs (Pollock et al., 1984). By growing a virus in the presence of neutralizing MAbs, escape mutants can be selected that are then sequenced to identify the substitution which rendered the mutant neutralization-resistant (Air and Laver, 1986; Wiley and Skehel, 1987).

Topographic Epitope Mapping

Competitive bindings assays with pairs of MAbs can be used to determine the relative position of epitopes on the surface of a protein (Berzofsky, 1984). However, two epitopes are recognized as different only if they are far enough apart to allow simultaneous binding of the two MAbs. Because of the bulkiness of antibody molecules, considerable steric hindrance may occur and MAbs directed against distinct but neighboring epitopes could thus be presented from binding simultaneously to the antigen surface. Although a single Fab antibody fragment covers about 700 Å^2 of the surface of an antigen, this does not mean that the epitope necessarily extends over the same area. Incidentally, this makes the argument for the exclusive existence of discontinuous epitopes in proteins (Barlow et al., 1986) less compelling, since a MAb recognizing,

for instance, a continuous epit pe of only three residues would still cover an area of about 700 Å² of the antigen surface.

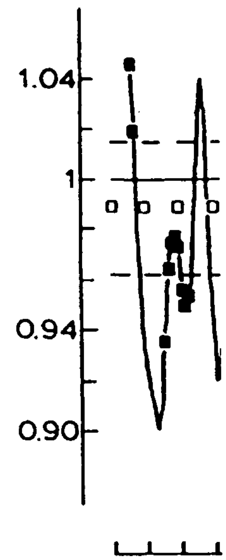
When the number of MAbs used in epitope mapping is large, one often observes a continuum of epitopes that can no longer be subdivided into discrete, separate antigenic domains (Mathews and Roehrig, 1984; Underwood, 1982). Instead of blocking binding, a competing antibody may actually enhance the binding of a second antibody presumably by an allosteric effect (Cecilia et al., 1988; Heinz et al., 1984). Such enhancement of binding may be caused by only one of two antibodies (unidirectional enhancement) or it may be bidirectional (Heinz, 1986).

THE PREDICTION OF CONTINUOUS EPITOPES

Many attempts have been made to correlate the location of continuous epitopes in a few well-characterized proteins with parameters such as the hydrophilicity, accessibility, and mobility of short segments of their polypeptide chains. All prediction calculations are based on propensity scales for each of the 20 amino acids. These scales describe the tendency of each residue to be associated with properties such as surface accessibility or hydrophilicity. Usually a window of seven residues is used in the analysis. The corresponding value of the scales is introduced for each of the seven residues and the arithmetical mean of the seven values assigned to the center of the window.

Various algorithms for predicting secondary structure have also been applied to the prediction of continuous epitopes. Whereas the core of proteins usually contains a combination of helices and sheets, their surface is replete with turns and loops (Rose et al., 1985a). The success rate of secondary structure prediction algorithms is limited since at most 55 to 70% of the structural elements are correctly predicted (Fasman, 1989; Kabsch and Sander, 1983). The predictive value of eight scales has been compared, using as a criterion of success the number of residues correctly predicted to be antigenic in four well-studied proteins (Van Regenmortel and Daney de Marcillac, 1988). It was found that none of the methods achieved a high level of correct prediction, although the hydrophilicity scale of Parker et al. (1986) and the segmental mobility scale of Karplus and Schulz (1985) were slightly more successful than the others (Fig. 3).

In a recent study, the validity of 22 different scales for predicting antigenicity was analyzed using 9 proteins containing 54 identified continuous epitopes (Pellequer et al., 1991). The method of analysis calculated how many residues of each protein were correctly or wrongly predicted to be antigenic. The results obtained with 10 of the scales are summarized in Table 2. From the ratio of correctly over wrongly predicted residues it was found that various hydrophobicity and hydrophilicity scales gave 51 to 57% correct predictions. The accessibility scales gave 46 to 52% correct predictions, whereas the scales that predict turns gave a slightly higher level of correct prediction (53 to



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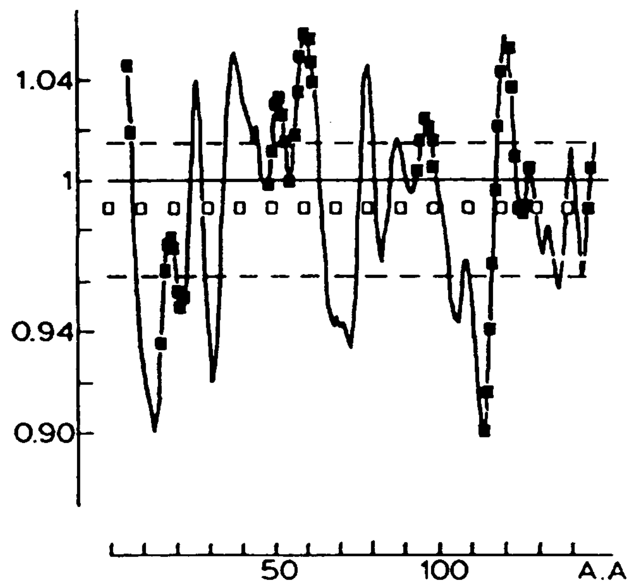

Figure 3. Prediction of continuous epitopes in proteins. Segmental mobility profile of myoglobin calculated with the scale of Karplus and Schulz (1985). The smoothing procedure of Van Regenmortel and Daney de Marcillac (1988a) was used. The black squares correspond to amino acids that are part of known continuous epitopes of myoglobin. The white squares represent the average value of the mobility parameter over the entire sequence and the two horizontal broken lines represent ±0.7 SD from the mean. Such an interval corresponds to 50% of the population.

61%). These results again confirm earlier studies (Getzoff et al., 1988; Van Regenmortel and Daney de Marcillac, 1988) which showed that none of the algorithms in current use give a high level of correct prediction.

CONCLUSION

The different analytical approaches used to delineate protein epitopes lead to different perceptions of the nature of protein antigenicity. The structural approach of X-ray crystallography concentrates on the relative position of atoms at the antigen-antibody interface and identifies epitopes as structural entities of 15 to 22 residues. In contrast, the functional approach based on cross-reactive binding measurements introduces the additional dimension of time and leads to the view that only about five residues are implicated in epitopes defined in a functional way. Furthermore, binding measurements are unavoidably submitted to operational constraints and different immunoassays or different types of probes (for instance, free peptide, conjugated peptide, or solid-phase peptide) point to different residues as being critical in an

TABLE 2.
Comparative Value f 10 Antigenicity Prediction Scales Applied t Nine Proteins of Kn wn Antigenic Structure

	Correctly Predicted Residues[a]	Wrongly Predicted Residues[a]	Ratio[b]	Percentage Correct Prediction[c]
Inverted hydrophobicity scale of Kyte and Doolittle, 1982	180	171	1.05	51
Inverted hydrophobicity scale of Rose et al., 1985b	150	134	1.11	53
Hydrophilicity scale of Hopp and Woods 1981	150	147	1.02	51
Hydrophilicity scale of Parker et al., 1986	190	145	1.31	57
Accessibility scale of Chotia, 1976	180	167	1.07	52
Acrophilicity scale of Hopp, 1984	142	124	1.14	53
Flexibility scale of Karplus and Schulz, 1985	152	138	1.10	52
Antigenicity scale of Welling et al., 1985	133	236	0.56	36
Scale for turns of Chou and Fasman, 1978	151	111	1.36	58
Scale for turns of Levitt, 1976	169	108	1.56	61

[a] The columns correctly predicted and wrongly predicted correspond to the number of correctly predicted and wrongly predicted amino acids, respectively, above the cut-off level (+ 0.7 × SE).
[b] Ratio of correctly predicted/wrongly predicted amino acids.
[c] Ratio [correctly predicted/(correctly predicted + wrongly predicted)] expressed as percentage correct prediction.

Adapted from Pellequer et al., 1991.

epitope. Structural and functional analyses have been termed "two different ways of seeing" (Lambert and Hughes, 1988), and these two approaches lead to complementary models of biological and immunological reality.

Epitopes are relational entities since they can be recognized only by the binding of complementary paratopes. An epitope is thus not an intrinsic feature of a protein molecule existing independently of its paratope partner. Epitopes and antigens can be defined only in terms of the emerging properties and relationships that arise in an immunological system.

The molecular dissection of protein antigens should thus not be confused with an attempt to reduce biology to chemistry. The distinction between an immunochemical interaction and a simple chemical one remains valid also when the binding reaction between two immunological reactants is analyzed in molecular terms.

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Predicti n Scales Applied
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›ngly ›licted idues[a]	Ratio[b]	Percentage Correct Prediction[c]
171	1.05	51
:34	1.11	53
.47	1.02	51
.45	1.31	57
.67	1.07	52
.24	1.14	53
.38	1.10	52
:36	0.56	36
.11	1.36	58
.08	1.56	61

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